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New Method for Estimating Digestion of *Paracoccidioides brasiliensis* by Phagocytic Cells In Vitro

MAURICIO GOIHMAN-YAHR,[1]* ERVIN ESSENFELD-YAHR,[2] MARIA C. ALBORNOZ,[1] LUIS YARZÁBAL,[1] MARIA H. DE GÓMEZ,[1] BLANCA SAN MARTÍN,[1] ANA OCANTO,[1] AND JACINTO CONVIT[1]

Pan American Health Organization Center for Research and Training in Leprosy and Related Diseases, Instituto Nacional De Dermatologia,[1] and Department of Pathology, Hospital "Padre Machado,"[2] Caracas, Venezuela

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We describe a method by which phagocytosis and digestion of *Paracoccidioides brasiliensis* yeast cells by polymorphonuclear leukocytes or other phagocytic cells may be estimated. Suspensions of *P. brasiliensis* in its yeastlike phase were sonicated, counted, and incubated with known numbers of peripheral blood polymorphonuclear leukocytes. At given intervals, cytocentrifuge droplets were stained by a variation of Papanicolaou's method. Stained preparations were examined with phase-contrast optics. Digested organisms showed total or partial disappearance of protoplasm. Green-stained cell walls resisted digestion. The proportion of digested cells as a function of time was estimated.

Paracoccidioidomycosis (South American blastomycosis) is a granulomatous disease. It is produced when *Paracoccidioides brasiliensis* grows in the tissues of susceptible individuals. Many organs or structures may be attacked. Among these are the lungs, mucosal membranes, lymph nodes, liver, spleen, and adrenals (4).

In the deep mycoses, digestion of fungi by phagocytic cells (such as the polymorphonuclear leukocytes [PMNs]) is of great importance. In the case of *Candida albicans*, for instance, digestion and intracellular killing may be estimated by a variety of methods. Known numbers of viable yeasts are put in contact with PMNs under appropriate culture conditions. At preset intervals, the fraction of yeast cells killed or digested is estimated. This may be done by liberating fungal cells from phagocytes and determining the percentage of live (or dead) yeasts. This percentage may be estimated by dye-exclusion methods or by calculating the number of viable particles as a function of the number of colonies obtained after plating (6). Intraphagocytic digestion may be evaluated by the microscopic features of intracellular yeasts as observed in Giemsa-stained preparations (5).

Interaction between *P. brasiliensis* yeast cells and PMNs has not been accurately explored by methods similar to those just outlined. This is so in part because of technical problems. Among these are the following: (i) *P. brasiliensis* is a dimorphic fungus. At body temperatures it grows in a yeastlike form. Suspensions may be obtained from solid or liquid media. Such suspensions are not homogeneous. The fungus grows in clumps formed by a highly variable number of cells of different sizes. Cells also produce varying numbers of buds. (ii) Plating efficiency of suspensions of *P. brasiliensis* has not been accurately determined, mainly because of problems in obtaining a precise initial particle count. (iii) The methods usually used to stain *P. brasiliensis*, as well as many other fungi (periodic acid-Schiff and Grocott methods, for instance), color the cell wall and not the protoplasm. As shown with *C. albicans*, digestion of the protoplasm is crucial for evaluating the destruction of phagocytosed fungal cells (5). Unfortunately, *P. brasiliensis* is not well stained by the Giemsa method.

We have devised a technique by which essentially single-cell suspensions of *P. brasiliensis* are obtained. Known numbers of yeastlike cells and phagocytic cells are incubated under appropriate conditions. Digestion of phagocytosed fungi is estimated by staining cytocentrifuge droplets with a variation of Papanicolaou's method. Stained droplets are observed with phase-contrast optics.

(An initial report of these findings was presented at the Annual Meeting of the Venezuelan Society for the Advancement of Science in 1977.)

MATERIALS AND METHODS

P. brasiliensis. We used two strains of *P. brasiliensis*. Strain Pb-9 was kindly provided by L. and G.

San-Blas (Instituto Venezoland de Investigacíones Científicas, Caracas, Venezuela). This is a nonpathogenic strain with a peculiar cell wall composition (10, 11). Strain 8506 was isolated by us from a case of paracoccidioidomycosis. Stock cultures of both were maintained in brain heart infusion agar slants (Difco) at 36.5°C. For experiments, gauze- and cotton-stoppered 50-ml Erlenmeyer flasks containing 15 ml of brain heart infusion plus 50 μg of gentamicin (reagent solution, Schering Corp.) per ml were consecutively inoculated every 3 to 4 days (late-log phase). Flasks were incubated in a reciprocating water bath shaker (Eberbach Corp.) at 33°C. Growth curves were estimated by direct particle counts (see below), and experiments were done with late-log-phase cells of *P. brasiliensis*.

Fungal suspensions. About 6 ml of culture fluid was obtained under sterile conditions. The suspensions were centrifuged (1,500 rpm in a GLC-2B General Laboratory centrifuge; radius, 16.41 cm) for 10 min and washed once with sterile saline. Single-cell suspensions were obtained by using ultrasound. Details of the method are described elsewhere (M. Goihman-Yahr, L. Pine, M. C. Albornoz, L. Yarzabal, A. Ocanto, M. E. Gomez, and J. Convit, Acta Cient. Venez. **28**[Suppl. 1]:118–119, 1977; Goihman-Yahr et al., manuscript in preparation). Briefly, 1-ml aliquots of the washed suspension were put in sterile, capped polystyrene tubes (12 by 75 mm). The tubes were kept standing in a specially designed holder, surrounded by ice water. Sonication was done with a 185 WD Branson Sonifier (Sonifier cell disruptor, model W185D; Heat Systems Ultrasonics Inc., Plainview, N.Y.), using a special microtip (Special Stepped Microtip, Heat Systems Ultrasonics Inc.) sterilized by flaming absolute ethanol. Sonication was carried out at a low intensity (scale no. 1 of the apparatus) for exactly 20 s. (Time and intensity of sonication were decided after extensive preliminary testing.) After vibration, the contents of the tubes were pooled and washed twice with saline. Fungal cells were counted in a hemocytometer. Counting was easier and the integrity of the fungal cells could be ascertained if phase-contrast optics were used (HI DLL Nikon objective with a Nikon phase-contrast turret condensor). The cells were centrifuged once more and resuspended in Hanks balanced salt solution (GIBCO) with 20% fetal calf serum (GIBCO), 10 U of sodium heparin per ml (Lilly), and 50 μg of gentamicin (reagent solution) per ml (HHGFCS). Concentrations were adjusted to either 10^7 yeast cells per ml (full strength) or 2×10^6 cells per ml (1/5).

Leukocytes. A 40-ml amount of fasting blood was drawn from a peripheral vein by means of a no. 19 butterfly infusion set. Blood was heparinized immediately (10 U of sodium heparin per ml) and mixed with dextran (6% Macrodex [Pharmacia] in saline; mean molecular weight, 70,000). One milliliter of dextran was used per 9 ml of blood. The mixture was sedimented at 36.5°C for 30 to 45 min. Additional blood was processed to obtain fresh autologous serum or else fresh frozen autologous serum was used. (Type AB Rh-positive pooled serum was used in other experiments with comparable results.) After sedimentation of the blood-dextran mixture, the upper layer was drawn off by means of a syringe with a hematocrit needle (pipette for Wintrobe tubes, no. 16; Clay-Adams). This layer was centrifuged (800 rpm for 10 min in a GLC 2B General Laboratory centrifuge), washed twice in Hanks-heparin-gentamicin, and finally resuspended in 3.2 ml of HHGFCS. Leukocytes were counted using standard counting solutions. Suspensions were adjusted with HHGFCS to contain 10^7 PMNs per ml. No hypotonic lysis was done. The viability of PMNs estimated by the erythrosin B method (8) was about 98%.

Mixtures. Incubation was done in sterile polystyrene tubes (12 by 75 mm). Each received a total of 1 ml, as follows: 0.25 ml of PMNs (10^7 per ml in HHGFCS), 0.25 ml of *P. brasiliensis* cells (10^7 or 2×10^6 per ml in HHGFCS), 0.25 ml of human serum (fresh or frozen, autologous), and 0.25 ml of Hanks-heparin-gentamicin.

The tubes were put in a rotating holder (Multipurpose Rotator, model 150V; Scientific Industries Inc., Springfield, Mass.) at 30 rpm and incubated for 1 and 2.5 h, respectively at 37°C. After each interval, samples were prepared by adding 2 drops of the incubation mixture and 6 drops of HHGFCS to the preparative chamber of a Shandon-Elliott cytocentrifuge (Shandon-Elliott Cytospin SCA-0030, Shandon Southern Instruments Ltd.). The chambers were rotated at 800 rpm for 10 min. Undried droplets were put in 95% ethyl alcohol in screw-capped Coplin jars. For control cytological purposes, duplicate droplets were air dried, fixed with methanol, and stained with Wright-Giemsa stain. Alcohol-fixed droplets were stained by a modification of Papanicolaou's method, as follows:

(i) Washed in running water for 1 min.

(ii) Stained with Harris hematoxylin for 2 min. (To prepare Harris hematoxylin, 10 g of hematoxylin [Merck] was dissolved in 50 ml of 95% ethanol. A 150-g portion of potassium alumina [kalium aluminum sulfate$\cdot$12H_2O, no. 1047, Merck] was dissolved in 3,000 ml of distilled water and heated. Once the alumina was dissolved, the hematoxylin was added slowly and the whole was allowed to boil. Six grams of red oxide of mercury [quecksilber(II) oxide rot, no. 4466, Merck] was added slowly. The whole was then quickly cooled. A 4-ml amount of glacial acetic acid was added to 96 ml of Harris hematoxylin before use.)

(iii) Washed again with running water for 1 min.

(iv) Differentiated (5 s each) with: (a) acid water (3 ml of hydrochloric acid [Merck] in 2,000 ml of distilled water). (b) running water. (c) ammoniated water (1 ml of concentrated ammonia in 400 ml of distilled water). (d) running water.

(v) Dipped in 50% ethanol for 1 min.

(vi) Dipped in 70% ethanol for 1 min.

(vii) Dipped in 80% ethanol for 1 min.

(viii) Stained in the following mixture for 3 min: 4 g of phosphotungstic acid (Wolframatophosphorsäure, no. 583, Merck); 2.5 g of eosin (eosin Gelblich, no. 1345, Merck); 1.5 g of orange G (orange G, no. 6878, Merck); 0.2 g of light green (light green SF [yellowish], color index no. 42095; total dye content, 65%; 254 Harleco); 0.3 g of Bismarck brown (Bismarck brown Y CI 21000, no. 458, Allied Chemical Corp.); 80 ml of distilled water; 920 ml of ethanol; 4 drops of a saturated solution

of lithium carbonate (May & Baker Ltd., Dagenham, England); (To prepare the mixture, each reagent was added separately to the 80 ml of water. This solution was added to the 920 ml of ethanol. Finally, 4 drops of lithium carbonate was added to the whole.)

(ix) Three washes with 95% ethanol, 1 minute each.

(x) One wash with absolute ethanol for 1 min.

(xi) One wash with alcohol/xylol (equal parts of each) for 1 min.

(xii) Three washes with xylol, 1 min each.

(xiii) Mounted in Permount (Fisher Scientific Co.).

Mounted preparations were examined with phase-contrast optics and oil immersion. Preparations stained with Wright-Giemsa stain were observed with standard optics.

The technique just described was routinely used. For control purposes, the following variations were used. (i) Live, sonicated *P. brasiliensis* were processed and incubated as described, but without PMNs. Samples were taken at different times from cytocentrifuge preparations. (ii) Suspensions of live *P. brasiliensis* yeast cells were processed and incubated with PMNs, but an initial sample was taken just after mixing the fungal cells and the leukocytes (T_0). (iii) Suspensions of live *P. brasiliensis* cells, growing in either solid or liquid media, were washed as described, but not sonicated. They were incubated without PMNs. Samples were taken at different intervals and processed and stained as described above.

Reading of preparations. Preparations were examined using phase-contrast optics. A reticulated eye piece (Bausch & Lomb micrometer disk) was helpful. At least 2 droplets and no less then 200 phagocytosed yeastlike cells were counted. The phagocytic index was determined in samples taken after 1 h of incubation.

RESULTS

Unaltered *P. brasiliensis* yeast cells showed a green cell wall and a brownish-orange patterned protoplasm. The shades of color varied according to the sample. Digested *P. brasiliensis* cells had a ghostlike appearance (G-cells); the cell wall surrounded in empty, faintly green space. Partially digested cells had a homogeneous, faintly colored protoplasm. The distinction between G-cells and normal cells was simplified by the use of phase-contrast optics. (As will be shown below, there was a small proportion of G-cells in the suspensions of the *P. brasiliensis* cells before incubation with PMNs.) The results obtained were expressed as a percentage of the G-cells (totally or partially digested) (see Fig. 1a and b).

Table 1 shows the percentage of G-cells in a representative experiment. Sonicated live *P. brasiliensis* 8506 were incubated with and without PMNs, and samples were taken at T_0 in addition to the usual intervals. It is clear that without PMNs the proportion of G-cells was low and did not increase with time of incubation. In contrast, this proportion increased considerably, as a function of time, when the *P. brasiliensis* cells were phagocytosed and digested by PMNs.

Table 2 summarizes the results of the tests done on 16 normal individuals (10 males, 6 females; mean age, 33.6 years; range, 21 to 47 years). The percentage of G-cells was shown as a function of time of incubation and of the proportion of PMNs per fungal cell. The phagocytic index was also determined. Both strains of *P. brasiliensis* were tested. Whereas individual results varied, it was apparent that the proportion of G-cells increased considerably when the fungi were incubated with PMNs (compare these results with those in Table 1). This proportion increased noticeably as a function of time of incubation and of the increased number of leukocytes per fungal cell. Cells from the nonpathogenic strain Pb-9 were digested more quickly than those of strain 8506. The results of the phagocytic index were concordant with the theoretical values expected from the relative proportions of PMNs and fungal cells.

Table 3 illustrates a representative experiment in which nonsonicated *P. brasiliensis* (growing in liquid or solid media) were incubated in the leukocyte-free mixture (the effects of leukocytes could not be studied in these preparations for the reasons outlined at the beginning of this paper). It is evident that even nonsonicated suspensions have a small percentage of G-cells. This proportion is somewhat lower, but in the same range, than that found in sonicated preparations (see Table 1). Thus, the initial small percentage of G-cells shown at T_0 in experiments such as that illustrated in Table 1 cannot be wholly attributed to sonication. As in the case of the latter suspension, this proportion of G-cells remained essentially unaltered throughout the experiment. The error in nonsonicated preparations was higher, because big clusters of *P. brasiliensis* yeast cells could not be adequately evaluated.

TABLE 1. *Proportion of P. brasiliensis (strain 8506) G-cells as a function of incubation time*[a]

Incubation time (h)	% of G-cells[b]	
	No PMNs in incubation medium	PMNs in incubation medium
0	10.3	9.2
1	8.5	25.0
2.5	6.3	37.1

[a] One *P. brasiliensis* yeast cell per PMN (2.5×10^6 yeastlike cells per tube).

[b] Percentage of G-*P. brasiliensis* yeast cells in stained droplets.

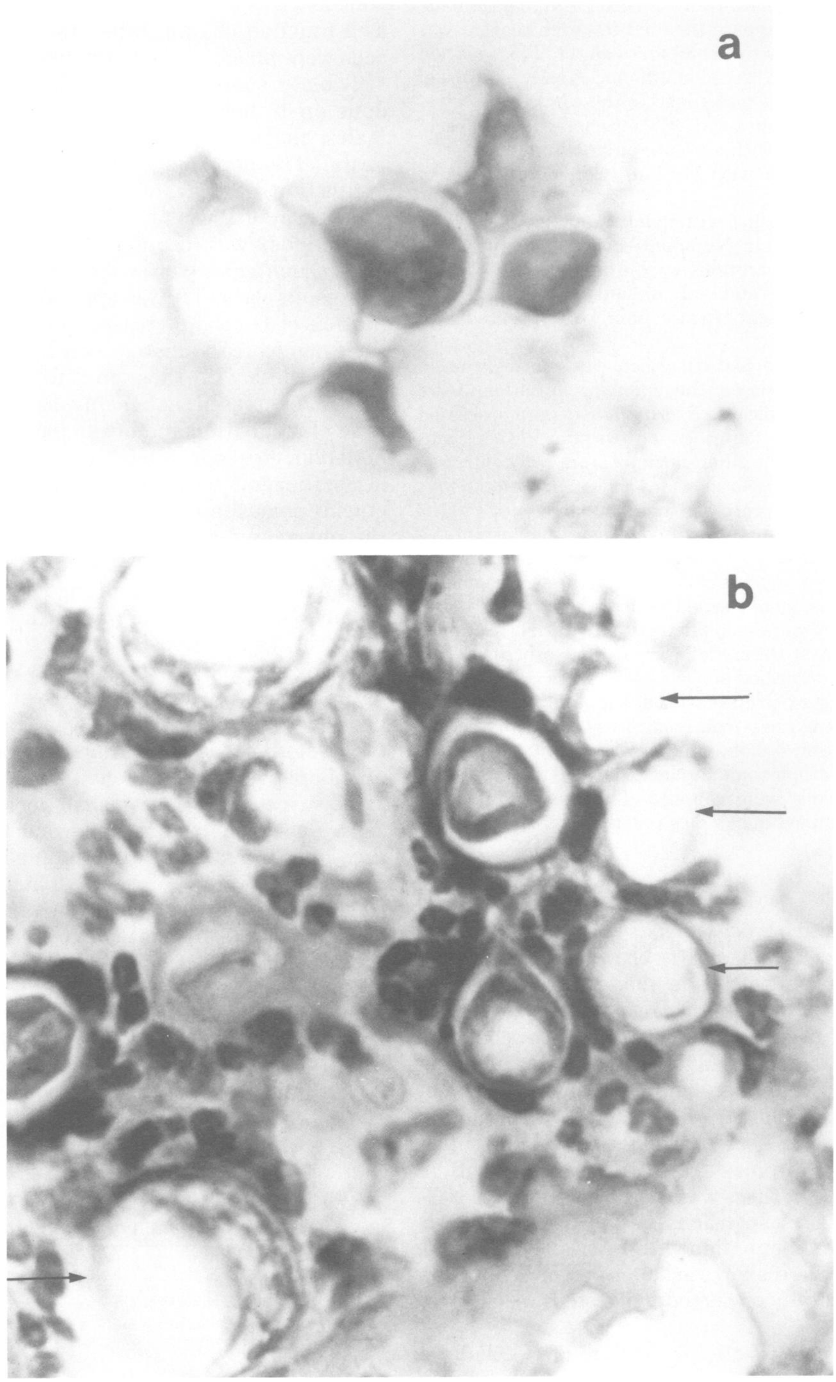


FIG. 1. *(a) P. brasiliensis incubated in medium with no leukocytes. Note integrity of protoplasm and the folded cell wall (modified Papanicolaou stain and phase contrast. Oil immersion.) (b) P. brasiliensis incubated with PMNs. The yeastlike cells are phagocytosed. Arrows indicate G-cells and yeasts partially digested. Other yeastlike cells are unaltered. (modified Papanicolaou stain and phase contrast, oil immersion.)*

TABLE 2. *Digestion of P. brasiliensis yeast cells by PMNs as a function of time of incubation, strain of fungus, and relative proportions of PMNs and fungal cells*

Strain	Phagocytic index[a]		% of cells[b] at incubation time:			
			1 h		2.5 h	
	FS[c]	1/5[d]	FS	1/5	FS	1/5
8506	0.86 (0.49–1.19)	0.23 (0.12–0.34)	24.04 (9.38–52.61)	44.42 (27.9–59.42)	39.24 (8.07–61.65)	57.83 (32.85–71.43)
Pb-9	0.86 (0.58–1.14)	0.23 (0.14–0.42)	37.69 (12.16–66.16)	57.43 (40.0–68.27)	47.71 (30.59–70.71)	63.23 (50.32–77.67)

[a] Mean phagocytic index after 1 h of incubation. Numbers in parentheses indicate the range.
[b] Mean percentage of G-*P. brasiliensis* cells in stained droplets. Numbers in parentheses show the range.
[c] FS, Full strength. One *P. brasiliensis* yeast cell per PMN (2.5×10^6 yeastlike cells per tube).
[d] One *P. brasiliensis* yeast cell per five PMNs (0.5×10^6 yeastlike cells per tube).

TABLE 3. *Proportion of G-P. brasiliensis cells as a function of time of incubation in medium with no PMNs (nonsonicated fungal suspensions)*

Strain and origin[a]	% of G-cells[b] at incubation time:		
	0 h	1 h	2.5 h
8506			
L	2.9	1.0	2.9
S	2.0	4.5	5.3
Pb-9			
L	3.6	4.5	4.2
S	6.7	3.1	6.1

[a] L, *P. brasiliensis* cells obtained from liquid medium (brain heart infusion); S, *P. brasiliensis* cells obtained from solid medium (brain heart infusion).
[b] Percentage of G-cells of *P. brasiliensis* in stained preparations.

DISCUSSION

The method described provides a useful tool for studying the interaction of *P. brasiliensis* yeast cells with phagocytic cells. Phagocytosis and intracellular digestion of this fungus can thus be estimated in vitro. Digestion of intracellular organisms is perhaps as important as killing. It has been used as a prognostic sign in granulomatous diseases such as leprosy (2). Studies comparable to ours have been done with *C. albicans* and other yeastlike organisms (5). The inherent characteristics of *P. brasiliensis* have hampered this line of research. A variation of Papanicolaou's stain was used by Miller et al. (7) to stain fungi shown in slide cultures. *P. brasiliensis* was not among those studied. No attempt was made by Miller et al. to estimate digestion of fungi. Sutliff and Cruthirds (13) used Papanicolaou's stain to detect *Blastomyces dermatitidis* yeast cells in sputum. The cytological features described resemble our findings with *P. brasiliensis*. Indeed, in Fig. 1c of Sutliff and Cruthird's paper are shown ingested *B. dermatitidis* cells with characteristics similar to those of our "G-cells." No correlation between cytological findings and digestion was indicated. The interaction between ingested *B. dermatitidis* and phagocytes was recently studied (12). The structure of *P. brasiliensis* cells in human tissue was described by Furtado et al. (3). Very recently, *P. brasiliensis* was found able to activate complement in vitro (1). Nonetheless, to our knowledge, the method described here is the first to allow an adequate study of the digestion of phagocytosed *P. brasiliensis* yeast cells in vitro.

We have not mentioned here the correlation between the proportion of digested cells and other criteria of viability (or lack of it), such as colony counts or use of vital stains (dye-exclusion methods). We were very successful in devising methods to improve plating efficiency. Initial results have been published (Goihman-Yahr et al., Acta Cient. Venez. **28**[Suppl. 1]:118–119, 1977). Nonetheless, plating efficiency is still relatively low (around 25%). Thus, experimental error is high enough to make such correlations impractical at the present time. Dye-exclusion methods have been used with *P. brasiliensis* (9). We have found such methods to be relatively trustworthy when used with *C. albicans*. However, they were not accurate enough when applied to *P. brasiliensis* yeast cells. Methylene blue was particularly unreliable.

The method described in this paper provides a relatively easy, reliable, and reproducible way of estimating the in vitro digestion of *P. brasiliensis* yeast cells by PMNs. It could be applied to other fungi and other phagocytic cells. Permanent preparations are obtained and may be stored for further reference. As in the case of *C.*

albicans (5), all of the ingested *P. brasiliensis* cells are not digested after phagocytosis. Digestion is a function of time and of the relative proportions of PMNs and fungal cells. We have shown evidence that a nonpathogenic strain is digested more quickly than a pathogenic one.

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